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A Flagellar Pocket Membrane Fraction from *Trypanosoma brucei rhodesiense*: Immunogold Localization and Nonvariant Immunoprotection

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In contrast to the abundance of detailed information on variant-specific surface coat antigens of African trypanosomes, data on possible common or nonvariant antigens within these protozoa are surprisingly limited. In this study, the cellular localization and protective potential of a previously characterized flagellar pocket membrane (FPM) fraction were determined. Immunogold staining of live trypanosome suspensions at 0 to 4°C by using anti-FPM hyperimmune serum raised in rabbits as the primary antibody revealed specific staining of the parasite surface at the emergence of the flagellum from the flagellar pocket. The same specificity of immunogold localization was obtained for each of three distinct variable antigenic types (VATs) of a serodeme of *Trypanosoma brucei rhodesiense* Wellcome strain. Products of translated mRNA preparations from each of the VATs were precipitated by the FPM antiserum and revealed identical banding patterns when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography. Immunization of mice with FPM fraction protected them against infection by two of the VATs; the third VAT was afforded poor protection. This is the first demonstration of the combined cellular localization, nonvariant nature, and protective potential of a membrane fraction from African trypanosomes.

Trypanosomiasis, or African sleeping sickness, is a parasitic disease, transmitted by the tsetse fly, which involves both humans and domestic animals over much of central and east Africa. People living in these regions are at constant risk, and past epidemics have struck rapidly and fatally, with mortality rates exceeding 30% in some areas. No immunoprophylactic treatment against trypanosomiasis is available for humans, and the few therapeutic drugs in use are extremely toxic.

The most immunogenic macromolecule found on the surface of bloodstream African trypanosomes is the variant-specific surface antigen, a glycoprotein that constitutes a 12- to 15-nm-thick layer or surface coat enveloping the parasite plasma membrane (1, 11, 21, 23, 26). As little as 3 μg of purified material confers protection to a mouse, but unfortunately the protection is only variable antigen type (VAT) specific (1). The ability of African trypanosomes to undergo antigenic variation by changing the variant-specific glycoprotein (VSG) composing the surface coat, coupled with an immune response that is selectively VAT specific, presents a major obstacle to immunization against trypanosomiasis.

Current interest in studies on the antigenic properties of African trypanosomes has focused almost exclusively on the phenomenon of antigenic variation, either on efforts to elucidate the mechanisms of expression and switching of genes coding for VSGs or on attempts to explain the lack of cross-reactivity by understanding the structural organization and topography of the VSGs (6, 13, 14). Unfortunately, results thus far have proven to be highly discouraging in that they serve to point out the seeming unattainability of controlling trypanosomes by means of vaccination. Even the supposedly limited number of metacyclic VATs does not offer promise for immunoprophylaxis, since the metacyclic-VAT repertoire is conserved neither over long periods of repeated cyclical transmission nor between epidemics (3).

In contrast to the abundant accumulation of information relating to VSGs, data on the subcellular distribution and properties of other antigens in African trypanosomes are surprisingly sparse. Certain components of bloodstream trypanosomes, such as structural proteins, enzymes, and membrane constituents, appear to remain unchanged during the course of infection. Although some of these components are responsible for cross-reactions during serological testing (12, 15), they have been reputed to show little immunogenic activity and have been considered poor candidates for vaccination experiments (4, 8, 24). However, such a determination is highly presumptive, since possible common or nonvariant antigens have not been sufficiently characterized to provide detailed information on their properties and protective potential.

Many external and internal antigens are shared by bloodstream forms and procyclic stages. Elimination of antibodies against these shared antigens from anti-bloodstream-form hyperimmune sera by adsorption with disrupted procyclic forms has enabled the demonstration of nonvariant antigens whose presence is limited to bloodstream forms (5). Quantitative immunofluorescence of living trypanosomes (5) and Western blot (immunoblot) analysis of whole-trypanosome antigen preparations (9) have revealed that small amounts of nonvariant antigens are present on the surface of bloodstream forms. A low-density membrane fraction, as opposed to the high-density surface membrane, has been described that contains seven antigenically active components, four of which appear to be glycoproteins (17) and two of which are intimately membrane associated with and exposed on the external (luminal) side of the flagellar pocket membrane (FPM) (19). Although the surface coat was originally described as being associated with the FPM, no evidence of

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any cross-reaction between surface coat and FPM antigens has been observed (17, 25).

The subcellular localization of FPM-associated antigens has been inferred from the distribution profiles of marker enzyme activities and relative fluorescence after fluorescamine surface labeling obtained from analytical cell fractionation procedures by using sucrose density gradient centrifugation (17). In view of the importance of the flagellar pocket as the principal site for pinocytotic nutrient uptake of exogenous protein molecules (7, 16), a more direct demonstration of these antigens as being of flagellar pocket origin would be desirable. Indeed, to ascertain the usefulness of FPM antigens as potential vaccine material, the possible nonvariant nature, the accessibility of the flagellar pocket as a target for antibody action, and the immunoprotective potential of FPM material also need to be determined.

Expecting that this minor class of surface components, the flagellar pocket antigens, will be antigenically conserved and persist as nonvariant antigens and that antibodies synthesized against FPM components will gain access to FPM antigens externally disposed at the opening of the flagellar pocket, we have begun a characterization of an FPM fraction from the Wellcome CT strain of *Trypanosoma brucei rhodesiense* with respect to its cellular localization, nonvariant or cross-reactive nature, and protective potential.

MATERIALS AND METHODS

Trypanosomes. Clones TRWR (Trypanosome Research Walter Reed) 1, TRWR 10, and TRWR 13 of *T. brucei rhodesiense* Wellcome strain, respectively referred to in a previous communication (21) as CP3B4, VAT 10, and VAT 13, were used in this study. TRWR 10 and TRWR 13 are antigenically unrelated to TRWR 1 and each other and represent cloned distinct antigenic types obtained from cloned TRWR 1, the latter having been obtained from, and bearing an antigenic relationship to, an isolate of the parent Wellcome CT strain. Details of cryopreservation as stabilates and the methods employed for isolation of trypanosomes from infected rats followed previously described procedures (21). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Institutes of Health, publication 85-23.

Isolation of FPM fraction. The FPM fraction was isolated from *T. brucei rhodesiense* (Wellcome CT strain) by a previously described procedure (17, 18) that included disruption of trypanosomes by grinding with glass beads, differential centrifugation of the homogenate, and separation of the particulate fraction on a discontinuous sucrose gradient.

Antibodies against FPM. Antibodies to purified FPM were raised in New Zealand White rabbits by following a procedure established in a previous report (19).

IGS. Isolated trypanosomes were pelleted (1,600 × *g* for 10 min) and suspended to a concentration of 10^8 parasites per ml in staining buffer (SB) consisting of 40 mM sodium phosphate (pH 8.0)–30 mM NaCl–1% glucose. Anti-VSG sera were in supply from a previous study (21). The immunogold staining (IGS) reagent used, GAR 20, is a goat anti-rabbit (immunoglobulin G) antibody complexed to 20-nm-particle-size colloidal gold and was purchased from SPI Supplies, West Chester, Pa. Indirect IGS was performed according to the following procedure. Portions (0.1 ml) of live trypanosome suspensions were added to 0.1-ml amounts of a 1:20 dilution of primary antibody (anti-VSG or anti-FPM) in SB. After incubation for 30 min on ice, the trypanosomes were pelleted, washed with 5 ml of SB, and suspended in 0.16 ml of SB. GAR 20 (0.04 ml) was added, the samples were incubated and washed as before, the pellets were suspended in 0.1 ml of SB, and the suspensions were fixed in 1.8% formaldehyde for 60 min on ice. Except for the fixation step, trypanosomes remained motile throughout the staining procedure.

Electron microscopy. IGS trypanosomes were examined in the transmission electron microscope (TEM) by negative staining techniques. One drop of a suspension of formaldehyde-fixed IGS trypanosomes was placed on the surface of 300-mesh Formvar-carbon-coated copper grids, and after excess fluid was withdrawn with the edge of a piece of filter paper the parasites were stained with either 0.1% or 1% sodium phosphotungstate, pH 7.0, for 30 to 60 s. The stain was then withdrawn from the grid with filter paper, and the grids were air dried before being examined in the TEM. Specimens were examined at 80 kV in the Siemens IA TEM at various magnifications, and the images were recorded on Kodak electron image plates.

Immunoprecipitation of translation products. [^{35}S]methionine-containing translation kits and En^{3}Hance were purchased from DuPont-New England Nuclear Corp., Boston, Mass.; Cronex intensifying screens for autoradiography were purchased from Du Pont Co., Wilmington, Del.; oligo(dT)-cellulose was purchased from Pharmacia P-L Biochemicals, Piscataway, N.J.; and reagents for RNA isolation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from GIBCO-Bethesda Research Laboratories, Inc., Gaithersburg, Md. Pansorbin was purchased from Calbiochem-Behring, San Diego, Calif., and washed three times with buffer D (27) before use. mRNA was isolated from 3-day bloodstream trypomastigotes by the method of Melton et al. (20) and purified over oligo(dT)-cellulose. Translations were performed according to the directions of the manufacturer by using a reticulocyte lysate system with [^{35}S]methionine as the radioactive tracer. Primary translation mixtures were diluted with buffer D, preabsorbed three times with Pansorbin, and reacted with antisera for a minimum of 1 h at 4°C. Immune complexes were collected with Pansorbin, washed six times with buffer G (27), and displayed on SDS-polyacrylamide gels treated with En^{3}Hance before autoradiography.

Immunoprotection. The antigenic ability of FPM to induce immunoprotection in ICR mice was tested by a previously described procedure (21). One volume of Freund complete adjuvant was emulsified with an equal volume of FPM. Each mouse was injected intraperitoneally with 0.1 ml of emulsion containing 25 μg of FPM protein. Mice were injected on days 0, 7, 14, and 21. Control mice received an emulsion of Freund complete adjuvant and distilled water. Fourteen days after the last injection, mice were each challenged with 1,000 highly motile trypanosomes obtained from thawed stabilates.

RESULTS

Immunoelectron microscopy. Figure 1 shows a scanning electron micrograph of *T. brucei rhodesiense*. The marked depression of the parasite surface surrounding the attachment of the flagellum outlines the underlying flagellar pocket. The remainder of the surface is featureless except for occasional folds or ridges.

Indirect IGS of live trypanosome suspensions by using homologous anti-VSG serum as the primary antibody re-

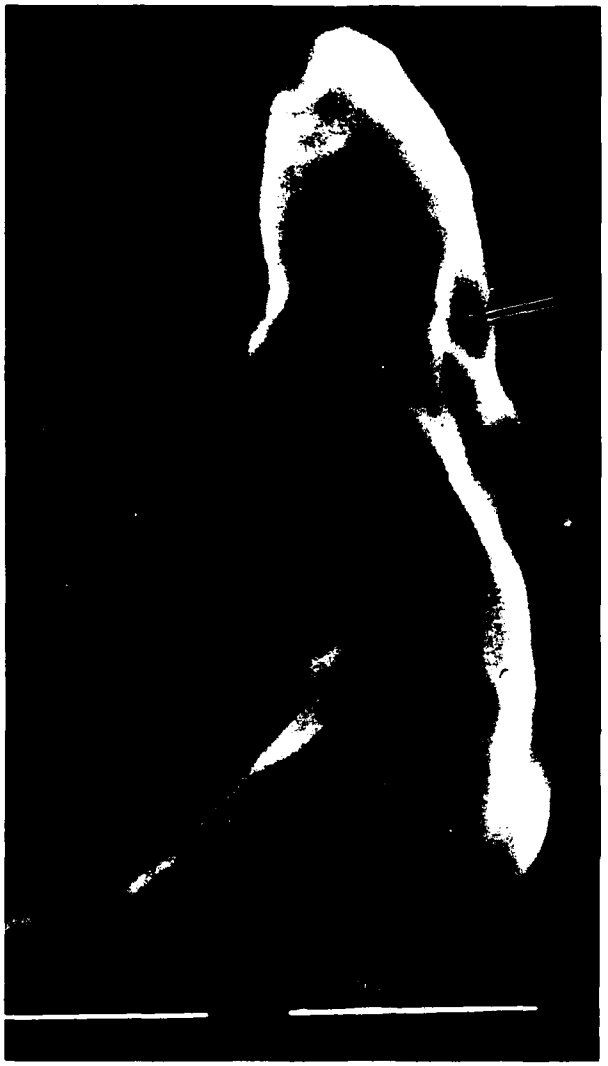

FIG. 1. Scanning electron micrograph of *T. brucei rhodesiense* (Wellcome strain) clone TRWR 1. Arrow points to surface depression that outlines the flagellar pocket. Bar, 1.0 μm.

vealed a deposition of sharply delineated, but aggregated or clustered, gold particles on the parasite surface (Fig. 2a). Electron-dense gold particles were almost completely absent from the surface of trypanosomes when the primary antibody used for indirect staining was normal rabbit serum (Fig. 2b) or nonhomologous anti-VSG serum (Fig. 2c). Use of antiserum to FPM material resulted in a strikingly specific interaction with the parasite surface (Fig. 3a and b). An almost exclusively localized encirclement of the flagellar pocket opening by the immunogold probe was observed. Indirect IGS of VATs TRWR 1 and TRWR 13 by FPM antiserum under identical conditions resulted in the same specificity of interaction and localization.

Immunoprecipitation of translation products. Anti-FPM serum was used to immunoprecipitate products in translated mRNA preparations from VAT TRWR 13. Analysis of immunoprecipitates by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 4, lanes 1 and 2) revealed four prominent bands with M_rs of approximately 80,000, 74,000, 40,000, and 25,000. Products of translated mRNA preparations from VATs TRWR 1 and TRWR 10 were also precipitated by the FPM antiserum and revealed identical banding patterns. Homologous anti-VSG antibody yielded only a single band of 55,000 M_r (Fig. 4, lane 3).

Immunoprotection against infection. The protective potential of FPM was tested by determining whether immunity against trypanosome infection could be produced in mice. Immunized mice received a total of 100 μg of FPM antigen over a period of 3 weeks and were challenged with 1,000 trypanosomes. The results listed in Table 1 provide evidence for the protective activity of FPM. Mice infected with TRWR 1 and TRWR 10 were afforded a measure of protection to the extent of 60 and 40% survival, respectively. Infection with TRWR 13 was lethal, and death occurred within 7 days. None of the nonimmunized control mice survived infection by any of the challenge VATs.

DISCUSSION

All members of the order *Kinetoplastidae* have a deep invagination of the surface membrane where the flagellum emerges from the cell. Thus, a lumen-containing pocket, termed the flagellar pocket or reservoir, surrounds the origin of the flagellum and has been found to be the principal site of uptake of exogenous macromolecules (7, 16). As stated previously (18, 19), the obvious importance of the flagellar pocket in the uptake of nutrients makes this site an attractive target for antibody action.

The flagellar pocket is also involved in the internalization of surface antigens complexed with antibody. The aggregation and subsequent movement (capping) of surface antigen induced by antibody progresses from the cell surface into the flagellar pocket. Capping is temperature dependent, occurring optimally at 37°C but not at all at 0 to 4°C, and, in bloodstream African trypanosomes, requires a double (indirect) antibody procedure (2, 10). In this study, an indirect IGS technique has been used to demonstrate the localization of interaction of antiserum to *T. brucei rhodesiense* flagellar pocket antigens, but the staining procedure with live trypanosome suspensions was done at 0 to 4°C to preclude the arisal of any spurious results owing to capping.

IGS trypanosomes have been examined in a TEM by negative staining techniques. Except for folds or ridges, the negative stain showed the featureless surface appearance of the cell body. In cells presumed to be osmotically damaged, the negative stain penetrated into the interior; the nucleus, microbodies, and other cytoplasmic organelles were clearly seen. Indirect IGS by using anti-FPM as the primary antiserum revealed the selective deposition of a narrow line of gold particles on the parasite surface, outlining the opening of the flagellar pocket. This restricted localization of staining was observed for all three VATs. In contrast, a scattered distribution of gold particles was obtained with anti-VSG sera and the staining was VAT specific.

The nonvariable nature of FPM antigens, as presently revealed by the heterologous interaction (demonstrated by immunoelectron microscopy) of anti-FPM serum with three distinct VATs, has also been examined by anti-FPM precipitation of products in translated mRNA preparations from these different VATs. Immunoprecipitated translation products, analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, produced identical banding patterns for each VAT, thus further attesting to the nonvariant or conserved nature of FPM antigens. Although successive multiples of molecular weight appear to be represented by the bands, an absence of in vitro posttranslational

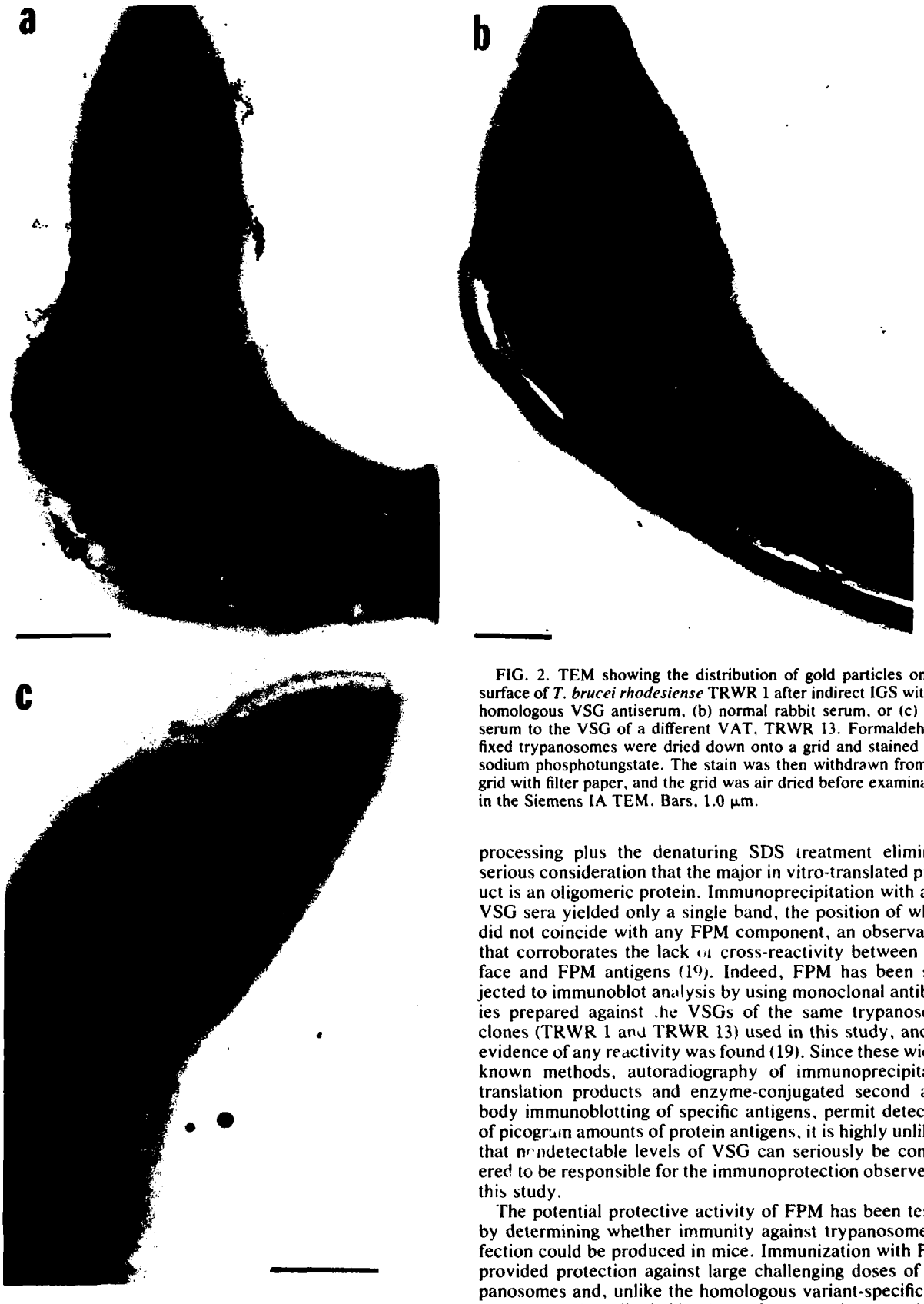

FIG. 2. TEM showing the distribution of gold particles on the surface of *T. brucei rhodesiense* TRWR 1 after indirect IGS with (a) homologous VSG antiserum, (b) normal rabbit serum, or (c) antiserum to the VSG of a different VAT, TRWR 13. Formaldehyde-fixed trypanosomes were dried down onto a grid and stained with sodium phosphotungstate. The stain was then withdrawn from the grid with filter paper, and the grid was air dried before examination in the Siemens IA TEM. Bars, 1.0 μm.

processing plus the denaturing SDS treatment eliminate serious consideration that the major in vitro-translated product is an oligomeric protein. Immunoprecipitation with anti-VSG sera yielded only a single band, the position of which did not coincide with any FPM component, an observation that corroborates the lack of cross-reactivity between surface and FPM antigens (19). Indeed, FPM has been subjected to immunoblot analysis by using monoclonal antibodies prepared against the VSGs of the same trypanosome clones (TRWR 1 and TRWR 13) used in this study, and no evidence of any reactivity was found (19). Since these widely known methods, autoradiography of immunoprecipitated translation products and enzyme-conjugated second antibody immunoblotting of specific antigens, permit detection of picogram amounts of protein antigens, it is highly unlikely that nondetectable levels of VSG can seriously be considered to be responsible for the immunoprotection observed in this study.

The potential protective activity of FPM has been tested by determining whether immunity against trypanosome infection could be produced in mice. Immunization with FPM provided protection against large challenging doses of trypanosomes and, unlike the homologous variant-specific immunoprotection afforded by the surface coat glycoprotein (1,

FIG. 3. TEM of indirect IGS *T. brucei rhodesiense* TRWR 10 by using antiserum to flagellar pocket membrane as the primary antibody. Trypanosomes were prepared for electron microscopy as described in the legend of Fig. 2. Bar equals 0.5 μm for panel a and 1.0 μm for panel b. Arrows point to the specific localization of gold particles on the cell surface where the flagellum emerges from the flagellar pocket.

11, 21), the protection obtained with FPM was heterologous, confirming previous preliminary observations (19). Since only one trypanosome is necessary to produce a parasitemia, a challenging dose of 1,000 was used to ensure an uncontrollable lethal infection (death by day 6 to 7). Indeed, almost all the mice in nonimmunized control groups succumbed by day 7. Immunized mice challenged with two distinct VATs, TRWR 1 and TRWR 10, resulted in three survivors of five tested and two survivors of five tested, respectively. No mice survived challenge with TRWR 13, and death occurred within 7 days.

Cloned isolates of antigenically distinct trypanosomes have been shown to have inherently different infectivities, growth rates, parasitemia profiles, immunosuppressive activities, and lethalities (22), all of which contribute to or correlate with parasite virulence. TRWR 10 and TRWR 13 are variant types obtained from a rabbit chronically infected with clone TRWR 1 of *T. brucei rhodesiense* Wellcome strain and correspond to cloned isolates of parasites in blood samples removed from the rabbit at days 25 and 45 postinfection, respectively. In contrast to TRWR 1 and TRWR 10, TRWR 13 has been observed (unpublished data) to be more strongly virulent, as evidenced by a shorter prepatent period, greater level of initial parasitemia, and shorter survival time of infected mice. Indeed, even immunization with surface coat antigen fails to protect against homologous infection with TRWR 13 (21). Accordingly, it is not altogether surprising that FPM antigen did not evoke heterologous protection against TRWR 13.

In a recent investigation, by using both crossed immunoelectrophoresis and immunoblotting, two principal and four

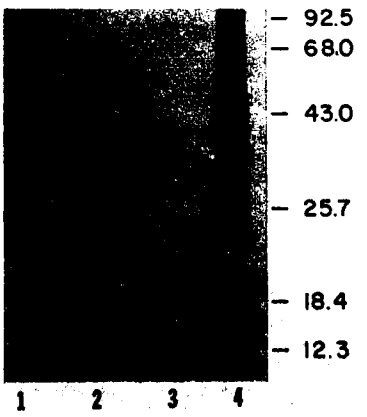

FIG. 4. Autoradiogram of SDS gel containing TRWR 13 mRNA-translated products immunoprecipitated by antiserum to FPM fraction (lanes 1 and 2) or to the surface coat VSG of TRWR 13 (lane 3). Lane 4 contains protein molecular weight standards. Lane 2 received one-half the amount of translated material received by lane 1.

TABLE 1. Immunoprotection of mice by an FPM fraction from challenge by three VATs of *T. brucei rhodesiense*

Immunization with[a]:	Challenge VAT	No. of mice dead by day[b]:										% Survival
		5	6	7	8	9	10	11	12	15	30	
FPM–FCA	TRWR 1	0	0	2	2	2	2	2	2	2	2	60
	TRWR 10	0	0	0	0	1	2	2	3	3	3	40
	TRWR 13	0	1	5	5	5	5	5	5	5	5	0
HOH–FCA	TRWR 1	2	4	5	5	5	5	5	5	5	5	0
	TRWR 10	0	3	4	5	5	5	5	5	5	5	0
	TRWR 13	0	0	4	4	5	5	5	5	5	5	0

[a] FCA, Freund complete adjuvant; HOH, distilled water.
[b] Each group contained a total of five mice.

minor components were established as authentic FPM antigens (19). Both of the principal antigens are glycoproteins, and surface labeling also has indicated these two antigens to be exposed at least partially on the external surface (luminal side) of the membrane. The present study has confirmed the exclusive association of FPM antigens with the flagellar pocket and has further demonstrated that they are nonvariable in nature, that they are accessible on the surface of live trypanosomes, and that they afford a measure of protection against challenge infection. Despite the enveloping layer of surface coat VSG, FPM components are sufficiently exposed to permit a narrow line of antibody interaction surrounding the flagellar pocket opening. It should be pointed out that antibodies also may have been directed to flagellar pocket protein antigens which were hidden from present immunoelectron microscopic viewing within the flagellar pocket of nonsectioned, but fixed and negatively stained trypanosomes. Whichever flagellar pocket antigenic component is involved, the interaction appears to have been adequate enough to effect protection in mice against challenge infection by two different VATs. However, strongly virulent strains that manifest general immunosuppression and decrease specific antibody production may pose problems in attempts to elicit immunity by FPM and other nonvariant antigens. Nevertheless, FPM material does appear to offer promise for immunoprophylactic application that bypasses the difficulty of antigenic variability.

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